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MOLECULAR CHARACTERIZATION OF EPIGEIC EARTHWORM EUDRILUS EUGENIAE (KINBERG), TREATED WITH TANNERY SLUDGE

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ABSTRACT

DNA barcoding is a powerful tool used for species identification by the expanding exploitation of mtDNA gene cytochrome c oxidase I (COI), as a genetic marker. In the present study, COI gene based DNA barcoding was employed to identify the epigeic species *Eudrilus eugeniae*. Molecular characterization of *Eudrilus eugeniae* was performed using Polymerase Chain Reaction (PCR) for COI gene. Phylogenetic tree was constructed using Molecular Evolutionary Genetics Analysis (MEGA7) software for the obtained sequences. The evolutionary divergences between its closely related species were also performed to disclose its amendments that occurred during evolution.

INTRODUCTION:

Research on taxonomy and biodiversity depends on efficient species diagnosis. DNA barcoding for animal species is based on exploitation of cytochrome c oxidase I (COI), which possesses high level of diversity. Mitochondrially based COI system of species identification provides much better taxonomic level of efficiency than morphological method of diagnosis [1]. DNA barcoding utilizes a short standard genomic portion referred to as 'DNA barcode', which has sufficient sequence variation to discriminate among various species to achieve unambiguous species level identification. The barcode may be constructed of one or several loci, together to be used as a complementary unit. One of the major advantages of DNA barcoding is that it provides 95% resolution of species-level recognition among mammals [2]. Also, it can be performed at any stage of life of an organism, whether live or dead [3].

Earthworms have the largest biomass (90%) among the soil invertebrates and constitutes a major component of the ecosystem. Disposal of sludge is an important yet tedious process in all industries. Among the cost-effective methods used for the treatment of sludge disposal, vermiculture is highly desirable. Eisenia foetida, Lampito mauritii and Eudrilus eugeniae together have been used in the biomanagement of papermill sludge [4]. The potentiality of these species was also assessed in terms of their efficiency and sustainability of vermicomposting water hyacinth, where Eudrilus eugeniae proved to be the most effective producer of vermicasts [5]. Identification of earthworm species is performed at an adult stage by dissection of the male genital organ [6], but that process is quite tedious, labor intensive and time consuming. Moreover, the other life stage worms remain unidentifiable with this method of identification. Hence, DNA barcoding is preferred as it applies to all life forms.

MATERIALS AND METHODS:

COLLECTION OF THE SAMPLE:

Epigeic species *Eudrilus eugeniae* (Kinberg) was used in the present study for the degradation of the tannery sludge. Earthworm was collected from Ramancherry, Chennai Tamil nadu, India in plastic containers with soil and cowdung, transferred carefully to the laboratory with adequate moisture. They were handsorted and the species were identified on naked morphological observations [7]. The biologically treated tannery sludge was collected from Pallavaram Tanners Association, Government Effluent treatment plant, Chennai Tamil Nadu, India.

EXPERIMENTAL SETUP OF VERMI REACTORS:

A Laboratory scale setup of vermi reactors was made up of plastic material in the form of open box provided with wire like holes at the bottom for drainage and ventilation. The reactors consisted of a basal layer of pebbles followed by coarse sand and a layer of garden soil and with a layer of dried cattle dung. These reactors were incubated with earthworms. The experimental units were maintained in triplicates and the control units were maintained five in numbers. In triplicates a total number of 36 experimental units were maintained to analyse the parameters at an interval of 12 days. The soil samples were collected from the triplicate experimental and control units of vermi composting reactors at an interval of 12 days. The soil samples and earthworm samples were subjected to physio-chemical parameters DNA barcoding and sequencing.

ISOLATION OF GENOMIC DNA:

Isolation of genomic DNA was carried out by phenol chloroform extration method [8]. The pellet obtained in the last step was washed with 70% ethanol, air-dried completely, suspended in Tris-EDTA buffer and stored at -20ºC until further use. The isolated DNA was assessed qualitatively and quantitatively by agarose gel electrophoresis and spectrophotometric method respectively.

PCR AND DNA SEQUENCING:

The polymerase chain reaction was carried out for amplification of the isolated DNA. The reaction volume was 20µl and the mixture consisted of forward and reverse primers, deoxyribonucleotide triphosphates (dATP, dCTP, dGTP and dTTP), template DNA sample and TaqDNA polymerase. Internal regions of COI gene were amplified using the following primers: COI-F(5'-GGTCAACAAATCATAAAGATATTGG-3') and COI-R(5'-TAAACTTCAGGGTGACCAAAAAATCA-3'). The reaction mixture was subjected to initial denaturation step at 94°C for 3minutes, followed by 35 cycles of denaturation at 94°C for 45 seconds, primer annealing at 47°C for 1minute, extension at 72°C for 1 minute and 20 seconds with the final extension at 72°C for 7 minutes. The PCR product underwent purification and DNA sequencing was performed by sanger sequencing (Applied biosytems 3500).

SEQUENCE ANALYSIS:

Nucleotide blast (BLASTn) using BLAST program and Genbank nucleotide database with default parameters was performed to determine the identity and the closest known relatives of the obtained sequences. Phylogenetic tree was constructed using maximum likelihood method in MEGA version-5 (Molecular Evolutionary Genetics Analysis). The Distance Matrix Explorer, an action menu of MEGA5 was used to compute the pair wise difference between the obtained target sequence to its maximum aligned sequence.

RESULTS:

DNA was isolated from the earthworm sample and its purity was quantified by the A260/A280 ratio in a spectrophotometer. DNA concentration along with the purity details are given in Table 1.

Table 1: Purity of the DNA observed in a spectrophotometer by calculating A260/A280 ratio.

Sample	Absorbance at 260nm(A260)	Absorbance at 280nm(A280)	Concentration (ng/µl)	Purity (A260/A280)
Sample 2	0.128	0.070	6400	1.82

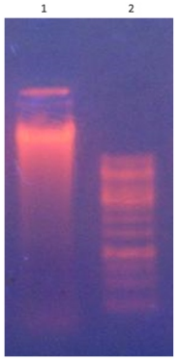

Fig. 1: Genomic DNA isolation *Eudrilus eugeniae*.
Lane 1: DNA from *Eudrilus eugeniae*, Lane 2: 1 Kb DNA Ladder

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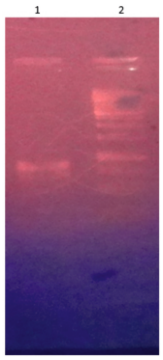

Fig. 2: COI gene amplification.
Lane 1: COI from *Eudrilus eugeniae*, Lane 2: 1Kb DNA Ladder.

Amplicons after PCR amplification were of 600bp for COI gene. Present study was conceded to determine the evolutionary relationship of *Eudrilus eugeniae.* The sequence obtained from the purified PCR product was subjected to comparison with the nucleotide database BLASTn and was found to have maximum identity to *Vignysa teres*, belonging to the family *Hormogastridae*. Construction of relationships between sequences is achieved using phylogenetic analysis that is aimed at identifying species and sequences from unknown samples [9]. Target, *Eudrilus eugeniae* branches to *Vignysa teres*, indicating the closest relationship, while it is distantly related to Megascolecidae species.

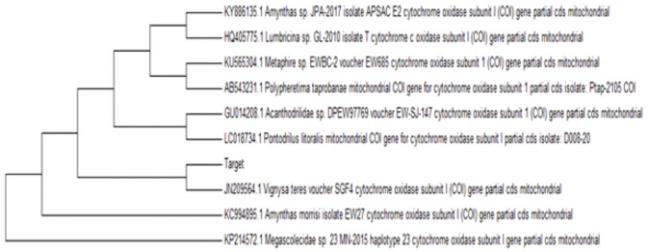

Fig. 3: Molecular Evolutionary Genetics Analysis of target Eudrilus eugeniae with sequence producing significant alignment from database for COI region.

Eudrilus eugeniae voucher SGO U2 2015 cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrial

Sequence ID: KX832073.1 Length: 644 Number of Matches: 1

Range 1: 25 to 636 GenBank Graphics

Score	Expect	Identities	Gaps	Strand
1120 bits(606)	0.0	610/612(99%)	0/612(0%)	Plus/Plus

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Query  1    TATAAGGCTTCTTATTCGAATTGAACTAAGACAGCCGGGTGCTTTTCTAGGAAGAGACCA  60
Sbjct  25   TATAAGGCTTCTTATTCGAATTGAACTAAGACAGCCGGGTGCTTTTCTAGGAAGAGACCA  84
Query  61   ACTCTATAACACTATCGTTACAGCTCATGCTTTTCTAATAATCTTTTTCCTTGTAATGCC  120
Sbjct  85   ACTCTATAACACTATCGTTACAGCTCATGCTTTTCTAATAATCTTTTTCCTTGTAATGCC  144
Query  121  AGTTTTTATTGGCGGATTTGGAAATTGATTACTCCCACTAATACTGGGAGCGCCCGACAT  180
Sbjct  145  AGTTTTTATTGGCGGATTTGGAAATTGATTACTCCCACTAATACTGGGAGCGCCCGACAT  204
Query  181  AGCATTCCCCCGACTAAATATTTTAAGATTTTGATTATTACCTCCTTCACTAATTCTCTT  240
Sbjct  205  AGCATTCCCCCGACTAAATAATTTAAGATTTTGATTATTACCTCCTTCACTAATTCTCTT  264
Query  241  AGTTTCGTCAGCTGCAGTTGAAAAGGGTGCAGGTACAGGATGAACTGTTTACCCACCACT  300
Sbjct  265  AGTTTCGTCAGCTGCAGTTGAAAAGGGTGCAGGTACAGGATGAACTGTTTACCCACCACT  324
Query  301  TGCAAGAAATCTTGCTCATGCGGGGCCCTCAGTAGACCTAGCCATTTTCTCTCTTCATCT  360
Sbjct  325  TGCAAGAAATCTTGCTCATGCGGGGCCCTCAGTAGACCTAGCCATTTTCTCTCTTCATCT  384
Query  361  TGCAGGGGCATCATCTATTTTAGGGGCAATTAACTTTATTACAACAGTAATTAATATACG  420
Sbjct  385  TGCAGGGGCATCATCTATTTTAGGGGCAATTAACTTTATTACAACAGTAATTAATATACG  444
Query  421  ATGATCCGGTCTTCGATTAGAACGAATTCCACTATTTGTATGAGCAGTAGTAATCACTGT  480
Sbjct  445  ATGATCCGGTCTTCGATTAGAACGAATTCCACTATTTGTATGAGCAGTAGTAATCACTGT  504
Query  481  AGTGCTACTTCTTCTATCACTTCCAGTCTTAGCGGGGGCAATCACAATACTTCTCACAGA  540
Sbjct  505  AGTGCTACTTCTTCTATCACTTCCAGTCTTAGCGGGGGCAATCACAATACTTCTCACAGA  564
Query  541  TCGTAATCTCAATACTTCTTTCTTCGACCCAGCTGGGGGTGGAGATCCAATTCTTTATCA  600
Sbjct  565  TCGTAATCTCAATACTTCATTCTTCGACCCAGCTGGGGGTGGAGATCCAATTCTTTATCA  624
Query  601  ACATTTATTCTG  612
Sbjct  625  ACATTTATTCTG  636
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Fig. 4:

DISCUSSION:

DNA barcodes have been proposed as a faster approach to provide novel species identification and discovery, unlike the taxonomists who would have spent decades to discover 10-15 million species using current description and publications [10]. The highly conserved mitochondrial enzyme cytochrome oxidase c is coded by multiple genes containing regions that evolved at different rates [11]. These markers were exploited as DNA barcodes because of their potential to identify putative regulatory elements, as they possess sufficient sequence diversity, individually or in combination, to discriminate among species [12]. The usual and recommended method of DNA barcoding involves polymerase chain reaction (PCR) amplification of suitable regions of the genome, sequence analysis of the amplicons, and alignment of the analysed sequence with reference sequences [13].

Figure 3 exemplifies the Maximum likelihood tree constructed for the target Eudrilus eugeniae using MEGA7 software. The evolutionary history was

inferred by using the Maximum Likelihood method based on the Tamura-Nei model [14]. The tree with the highest log likelihood (-6600.24) is shown. The bootstrap consensus tree inferred from 500 replicates is taken to represent the evolutionary history of the taxa analyzed [15]. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 10 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 595 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 [16]. The genomic analysis should that there were no major changes in nucleotide sequences after the earthworm was treated with tannery sludge for vermicompost.

CONCLUSION:

DNA barcoding has great potential for enhancing ecological and evolutionary investigations if the right genetic markers are exploited. In addition, phylogenetic analysis is also important for identification of the potential changes in the nucleotides of a species and its variations. In the present study, molecular characterization of the earthworm *Eudrilus eugeniae* was carefully studied using COI gene and its evolutionary relationship was constructed using MEGA 7.

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